Plant Cell Reports (2000) 19:224–228

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Transient gene expression in pine pollen tubes following particle bombardment

Received: 17 December 1998 / Revision received: 17 March 1999 / Accepted: 14 April 1999

Abstract A biolistic particle delivery system was used to genetically transform pollen tubes of three species of white pine (*Pinus aristata*, *P. griffithii* and *P. monticola*). The introduced plasmid DNA contained the GUS coding sequence flanked by the 35S CaMV promoter and NOS terminator sequences. Successful gene delivery was demonstrated by transient GUS expression as evaluated by standard histochemical assay. Distance of target specimens significantly influenced transient GUS expression in all three species of white pine. A target distance of 6 cm resulted in a significant number of transformed pollen tubes in *P. aristata* and *P. griffithii*, while distances of 6 and 9 cm resulted in a significant number of transformed pollen tubes in *P. monticola*. Generally, the number of pollen tubes expressing GUS activity was higher in *P. aristata* than in *P. griffithii* and *P. monticola*. The possibility of using GUS-transformed pollen tubes in conjunction with in vitro fertilization in conifers was examined. Gene expression in pollen tubes was also examined under electron microscopy where the X-glu reaction product occurred as large crystalline electron-dense precipitates in the cytoplasm.

Key words *Pinus aristata* · *P. griffithii* · *P. monticola* · Pollen tubes · Transformation · Particle bombardment · In vitro fertilization

Communicated by F. Constabel

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Abbreviations *GUS*: β-Glucuronidase · *CaMV*: Cauliflower mosaic virus · *NOS*: Nopaline synthase · *X-glu*: 5-Bromo-4-chloro-3-indolyl-β-D-glucuronide

Introduction

Genetic transformation in conifers has been most extensively studied in pines (Sederoff and Stomp 1993). DNA transfers have been carried out to test various methods of introducing DNA, optimize transient expression protocols, and express marker gene sequences using a variety of promoters and tissues (Sederoff et al. 1986; Gupta et al. 1988; Morris et al. 1989; Tautorus et al. 1989; Stomp et al. 1990; Campbell et al. 1992; Loopstra et al. 1992). Stable transformation has been achieved in *Pinus radiata* (Walter et al. 1998).

In pines, a rapidly developing area of interest is particle-mediated pollen transformation. Work has not only been done to optimize bombardment parameters and test marker and promoter genes (Hay et al. 1994; Martinussen et al. 1995; Tian et al. 1997), but also to use transformed pollen grains for conventional pollinations and subsequent recovery of transgenic progenies (Häggman et al. 1997; Aronen et al. 1998). Pollen grains are natural vectors for gene transfer because they are involved in sexual reproduction. Therefore, pollen transformation may also be extended to in vitro fertilization, a new development that promises a novel approach to conifer breeding (Fernando et al. 1998). One of the immediate needs of conifer in vitro fertilization is a rapid system to confirm the occurrence of fertilization in vitro. Currently, the work involves tedious histological processing (Fernando et al. 1997, 1998). Through the co-culture of transformed pollen tubes with untransformed female gametophytes, the resulting progenies can be easily assayed for the expression of foreign genes. Alternatively, transformed eggs may also be co-cultured with untransformed pollen tubes.

As part of ongoing work on in vitro fertilization in pines (Fernando and Owens 1998), this paper reports for the first time, the transient expression of the bacterial β-glucuronidase gene in pollen tubes of three species of pine, *Pinus aristata* Engelm. (bristlecone pine), *P. griffithii* McClelland (blue pine), and *P. monticola* Dougl. (western white pine). *Pinus aristata* and *P. griffithii* have been ranked by Bingham (1983) as two of the most resistant species to white pine blister rust (*Cronartium ribicola* J.C. Fisch. ex Rabenh.). Therefore, these pines are ideal for use in breeding with *P. monticola*, a species that is very susceptible to *C. ribicola* but highly valued for its fast growth and excellent lumber quality (Bingham 1983). We also examined the possibility of using transformed pollen tubes in conjunction with conifer in vitro fertilization and described the product of X-glu reaction as it appears under light and electron microscopy.

Materials and methods

Plant materials

Pollen cones of *P. aristata* were obtained from the University of Victoria, British Columbia, while pollen cones of *P. monticola* were obtained from Saanich Seed Orchard, Saanich, British Columbia. Pollen cones from both species were collected 2–3 days before dehiscence and surface-sterilized by washing with freshly prepared 70% ethyl alcohol, sterile distilled water, and freshly prepared 1% sodium hypochlorite for 30 s each step. Pollen cones were rinsed three times with sterile distilled water for 10 s each step and blotted dry on sterile paper towels. They were left in a petri dish for 72 h at 27 °C. During this time, the petri dish covers were replaced with sterile Whatman filter papers (11 cm) and fastened with rubber bands to ensure sterility during drying and shedding of pollen grains. Dried sterile pollen grains were collected and kept in 10-ml sterile screw-cap vials at 4 °C until used. This method is modified from Friedman (1987). Pollen grains (non-sterile) of *P. griffithii* were obtained from the Institute of Forest Genetics, Placerville, California.

Media composition and culture conditions

The pollen germination medium contained 100 μg/ml boric acid and 300 μg/ml calcium nitrate supplemented with 145 m*M* sucrose and 0.4% phytagel. The pH was adjusted to 5.8. Pollen grains were grown and bombarded on this medium and then incubated in the dark at 27 °C.

Particle bombardment

Pollen grains of each of the three species were evenly dispensed by means of gently shaking a sterile spatula containing pollen grains over nylon membranes (Gelman Sciences, Mich.). Approximately 50 mg of pollen grains were dispensed per nylon membrane. This method allows for a single to a few layers of pollen grains on the membranes. Similar pollen lots were used in all trials. Pollen grains were bombarded 1 h after plating.

DNA incorporation was carried out using the Biolistic PDS-1000/He System (Bio-Rad). All accessible parts of this equipment were surface-sterilized using 70% ethyl alcohol 15 min before use. Optimum bombardment conditions were performed with a rupture disk pressure of 900 psi, a gap distance of 1.5 cm, a macrocarrier travel distance of 8 mm, and target distances of 6,9 and 12 cm.

The plasmid DNA used was pBI221 (5.7 kb) containing the bacterial GUS gene inserted between the CaMV 35S promoter and NOS terminator sequences (Jefferson 1987). Plasmid DNA (10 μg) was precipitated on 3 mg 1.5- to 3.0-μm-diameter gold microcarrier particles (Aldrich Chem. Co, Wis.) following the protocol of Jefferson (1987). Aliquots (10 μl) were immediately spotted onto macrocarriers (Bio-Rad), air-dried, and mounted in the pistol chamber. Approximately 1 μg DNA was introduced per bombardment. Pollen grains of all three pine species were bombarded with plasmid DNA. Similar samples of all three species that were not bombarded and bombarded with gold particles without DNA served as the controls. Treatments were replicated three times.

GUS histochemical assay

After bombardment, petri dishes were sealed with parafilm and placed in a dark growth chamber at 27 °C for 48 h. The nylon membranes containing pollen grains were gently transferred into a petri dish containing a filter paper saturated with 1 ml GUS reaction buffer (0.1 *M* sodium phosphate buffer, 10 m*M* EDTA, 0.5 m*M* potassium ferricyanide, 0.5 m*M* potassium ferrocyanide, 1 m*M* X-glucuronide, 0.1% triton X-100, pH 7.0) (Stomp 1992). These were incubated in the dark for 24 h at 27 °C. The number of pollen tubes expressing GUS activity was counted immediately after 24 h. Results were expressed as the number of transformed pollen tubes per bombardment.

Histological analysis

Pollen tubes were fixed in freshly prepared 4% glutaraldehyde in cacodylate-sucrose buffer (200 m*M* cacodylate buffer and 145 m*M* sucrose), rinsed with cacodylate-sucrose buffer, dehydrated through a graded series of ethyl alcohol, and gradually embedded in Spurr's resin (Spurr 1969). Thin sections were cut using a Reichert-Jung Ultracut E ultramicrotome. Unstained sections were examined under light and transmission electron microscopy (Hitachi H-7000) at 75 kV.

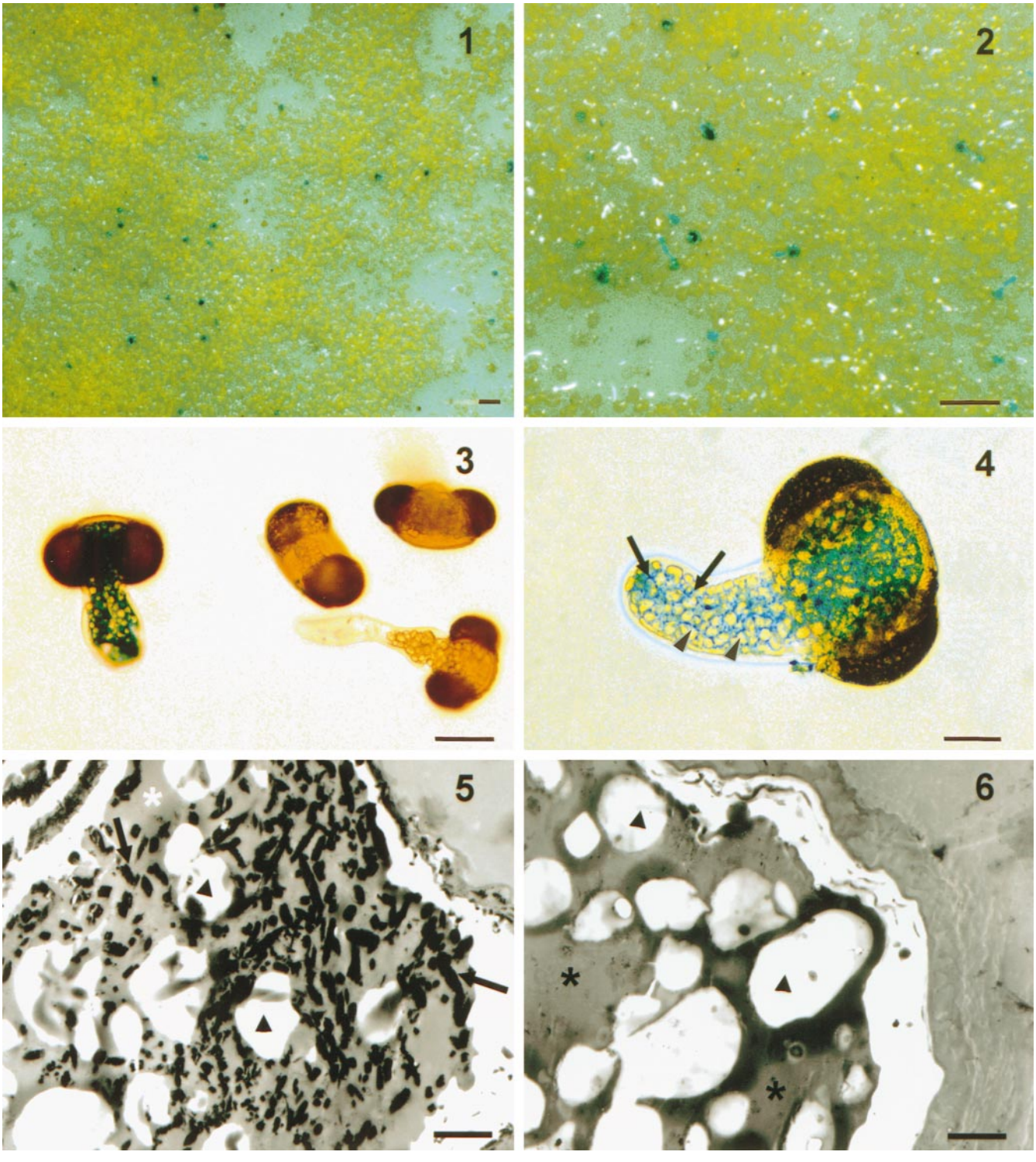
Statistical test

One-way ANOVA procedures were performed following natural log transformation. Differences were detected with Tukey's Honestly Significant Difference Test using SPSS (SPSS 1995). This software was also used to calculate means and standard errors (SE).

Results and discussion

Enzymatic cleavage of the histochemical substrate X-glu as manifested by the appearance of blue precipitates indicate that the bacterial β-glucuronidase gene was transiently expressed in the pollen tubes of all three species of pine examined (Figs. 1, 2 and 3). The number of pollen tubes in each species expressing GUS activity is shown in Table 1. Most of the pollen grains had germinated after 24 h. No GUS activity was found in pollen grains not bombarded or bombarded with gold particles without DNA. Faint background GUS activity was observed only in those pollen tubes left in GUS reaction buffer for more than 1 week.

The number of transformed pollen tubes was generally higher in *P. aristata*, followed by *P. monticola* and then *P. griffithii* (Table 1). In all three species of pine,



Figs 1–6 Expression of the GUS gene in pollen tubes of three species of pine. **1** GUS activity as manifested by the formation of blue precipitates in some pollen tubes of *P. monticola*. *Bar:* 1.5 mm. **2** Pollen tubes of *P. aristata* following a 24-h incubation in X-glu. *Bar:* 700 μm. **3** Two germinated and two ungerminated pollen grains of *P. griffithii*. Transformed pollen tube on *left*. *Bar:* 40 μm. **4** Blue precipitates in cytoplasm (*arrows*) surrounding starch grains (*arrowheads*) in *P. aristata* pollen tube. *Bar:* 20 μm. **5** Electron micrograph of an unstained section of a GUS-transformed pollen tube of *P. aristata* showing crystalline electron-dense precipitates (*arrows*) and starch grains (*arrowheads*) in the cytoplasm (*asterisk*). *Bar:* 4 μm. **6** Electron micrograph of an unstained section of an untransformed pollen tube of *P. aristata*. *Bar:* 3 μm

Table 1 Number of pine pollen tubes expressing GUS activity at varying target distances

Target distance (cm)	*Pinus aristata*				*Pinus griffithii*				*Pinus monticola*			
	Trials			Mean[a] (SE)	Trials			Mean[a] (SE)	Trials			Mean[a] (SE)
	1	2	3		1	2	3		1	2	3	
6	177	183	224	195a (±15)	79	86	35	67 (±16)	73	52	91	72a (±11)
9	103	68	123	98b (±16)	0	0	0	0	126	148	80	118a (±20)
12	0	0	0	0	0	0	0	0	29	20	42	30b (±6)

[a] Mean values followed by the same letter do not differ significantly ($\alpha = 0.05$)

significant differences were observed between the different distances of target specimens. A target distance of 6 cm resulted in a significant number of transformed pollen tubes in *P. aristata* and *P. griffithii*. None of the *P. aristata* pollen tubes was transformed at target distance of 12 cm, while none of the *P. griffithii* pollen tubes was transformed at target distances of 9 and 12 cm. In *P. monticola*, the numbers of pollen tubes transformed at target distances of 6 and 9 cm were not significantly different from each other but were significantly lower than those at a target distance of 12 cm. Target distance has a major effect on the efficiency of DNA delivery because as it is increased, the density of bombardment decreases (Heiser 1992). Since particle-mediated transformation appears as a statistical process, a shorter target distance maximizes the probability of hitting specimens. In other pines, target distance of 9.5 cm has been shown to produce the highest percentage of pollen transformation (Hay et al. 1994; Häggman et al. 1997).

Culture plates containing bombarded pollen grains of *P. aristata* and *P. monticola* were not contaminated even after 8 weeks of continuous incubation at 27 °C. Although surface sterilization of all possible components of the Biolistic PDS-1000/He System prevented the introduction of microbial contamination, the use of sterile pollen grains primarily determined the sterility of cultures. Using non-sterile pollen grains, as in the case of *P. griffithii*, contamination started to appear 48 h after incubation. This observation indicates that bombarded sterile pollen grains can be further grown in culture without the risk of contamination. Therefore, it is possible to use GUS-transformed pollen tubes in conjunction with work on in vitro fertilization in conifers.

The GUS histochemical test was previously regarded as a destructive assay (Sederoff and Stomp 1993). However, the rapidly accumulating reports on non-destructive assays are extending the usefulness of GUS as a reporter gene (Gould and Smith 1989; Martin et al. 1992a; 1992b; Stomp 1992; Kirchner et al. 1993). Overnight exposure to the GUS reaction buffer is non-toxic to plant cells if they are transferred onto a new medium (Martin et al. 1992b). Under a dissecting microscope, GUS-transformed pollen tubes are easy to identify, isolate, and transfer to another medium. Our preliminary results indicate that the GUS histochemical assay is also non-destructive to white pine pollen tubes. In contrast, pollen tubes transformed with the green fluorescent protein gene would be very difficult to isolate amidst the hundreds of untransformed pollen tubes because of the conformation of the fluorescent microscope. Also, the use of a selectable marker such as antibiotic resistance would not be feasible because of the need to co-culture untransformed female gametophytes in the same plate as the transformed pollen tubes. On the other hand, transferring antibiotic-transformed pollen tubes to another medium could damage pollen tubes.

Gene expression in pine pollen tubes was examined under light and electron microscopes. The identification of the X-glu reaction product under light and electron microscopy may aid in the interpretation of male cytoplasmic contents in egg cytoplasm when GUS-transformed pollen tubes are co-cultured with untransformed female gametophytes. Under light microscopy, the final insoluble product of the X-glu reaction occurs as blue precipitates that appear to completely fill the pollen-tube cytoplasm except in the areas occupied by starch grains (Fig. 4). Under electron microscopy, the X-glu reaction product occurs as crystalline electron-dense precipitates in pollen-tube cytoplasm (Fig. 5). Sizes of precipitates vary considerably, but those that are elongated have lengths ranging from 0.5 μm to 1.5 μm, and widths from 0.15 μm to 0.2 μm. No other type of precipitate or any indication of GUS activity was observed in any cellular compartment in the pollen tube. In untransformed pollen tubes, no crystalline electron-dense precipitate was observed (Fig. 6). Transformed and untransformed pollen tubes were processed under identical conditions including the elimination of post-fixation with osmium tetroxide and staining to avoid any possible misinterpretation that can be introduced by these reagents. In procambial cells of transgenic tobacco root (Craig 1992), the X-glu reaction product was observed in endomembrane systems and appeared as small electron-dense precipitates. Although the size was not reported by Craig (1992), the precipitates shown are obviously smaller than those observed in the cytoplasm of GUS-transformed pine pollen tubes. It is possible that the discrepancies in size may just be due to differences in concentrations or

quantity of GUS expression. In terms of where GUS expression usually occurs in transgenic plant cells, Iturriaga et al. (1989) have clearly pointed out that the GUS enzyme is localized in the cytoplasm unless a particular gene construct targets the enzyme to a subcellular compartment.

Acknowledgments We thank David R. Johnson for providing pollen grains of *P. griffithii*, Milan Osusky and Malinee Chaithai for isolating and sharing the plasmid DNA, Heather Down for organizing the figures, and Glenda Catalano for assisting in tissue processing for microscopy and photoprinting. This research was supported by a Natural Sciences and Engineering Research Council Operating Grant (A1982) to JNO.

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